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# Effects of Diethylstilbestrol, Norethindrone, and Mestranol on Selected Microbes

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# EFFECTS OF DIETHYLSTILBESTROL, NORETHINDRONE, AND MESTRANOL ON SELECTED MICROBES

by

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **Hormones** | |
| DS | diethylstilbestrol |
| DS di | diethylstilbestrol |
| MS | mestranol |
| NED | norethindrone |
| NED-MS | norethindrone and mestranol combined |
| PCS | plasma combination of steroids |
| **Media** | |
| THB | Todd Hewitt broth |
| TSB | tryptic soy broth |
| **Chemicals** | |
| ETH | ethanol |
| TCA | trichloroacetic acid |
| **Terms** | |
| CFU | colony forming units |
| CPM | counts per minute |

# I. REVIEW OF THE LITERATURE

The importance of the physiological functions of hormones in the human system cannot be overemphasized. The steroid sex hormones are important not only because of their natural functions, but also because of their clinical use. Diethylstilbestrol and diethylstilbestrol dipropionate are synthetic compounds with estrogenic activity. They are used extensively for estrogen deficient patients under the brand names "Des", "Stilbetin", etc.; and they are sometimes given as a post-coital oral contraceptive. Mestranol and norethindrone are combined commercially in an approximate ratio of 1 part mestranol to 10 parts of norethindrone. This preparation is sold under the name of "Norinyl" and is used widely as an oral contraceptive. Despite their importance, little is known of either the primary or secondary actions of these or other sex steroids. Much scientific research has recently focused in these areas.

Differences in physiological state due to sex or sex hormones have been implicated as factors in the infectious disease process. As early as 1920, pregnancy was found by Brown and Pierce (1920) to suppress experimental syphilis infections in animals, and as recently as 1973 sex was reported to affect the outcome of many experimental diseases in laboratory animals (Goble and Konopka, 1973).

Clinical studies have shown that such differences in physiological state affect host-parasite relationships in man. Pregnancy has been linked with an increased susceptibility to vaginitis (Carter, et al., 1940).

Washburn, et al., (1965) found sex differences in patients' susceptibility to bacterial meningitis and septicemia. Some clinical studies, such as that by Walsh, et al. (1968), have reported an increase in vaginitis caused by Candida albicans when patients used sex steroids as oral contraceptives. Yaffee and Grots (1965) reported that before four patients with severe vaginitis could be cured, the oral contraceptives they were using had to be discontinued. However, contradictory data on vaginitis has also been published. Spellacy, et al. (1971), found no increase in the incidence of vaginitis in women using oral contraceptives.

Experimental doses of sex steroids have been shown to alter the course of experimental infections in laboratory animals. One of the earliest such investigations was that by Frazier, et al. (1935), in which estrogens were found to affect the outcome of experimental syphilis in rabbits. In a study by Von Haam and Rosenfield (1942), the steroids estrone and estrogen protected laboratory animals against infection with virulent pneumococci. Foley and Aycock (1944) found that stilbestrol protected mice against experimental streptococcal infections. Mankowski (1954) demonstrated that mice were slightly protected against experimental infections by aspergillis when they received doses of testosterone. Yotis (1967) found that norethindrone had a protective effect on the development of staphylococcal lesions on rabbits.

Due to the complexity of the in vivo system, the actual factors involved in these effects on infections are not known. Factors such as steroid induced changes in the immune system, hormonally induced tissue changes, and

alterations in serum components have been hypothesized by various researchers. The influence of steroids on infection has also been attributed to a direct action by the steroids on the infectious agent. Many studies clearly show that sex steroids have an *in vitro* effect on some microorganisms. The importance of this effect in an *in vivo* situation is difficult to determine, but it is worthy of attention.

The actions of sex steroids on microbes has been studied in a number of *in vitro* growth experiments. Brownlee, *et al*. (1943), reported that diethylstilbestrol, estradiol, and estrone inhibited the growth of *Staphylococcus aureus* and *Streptococcus pyogenes* but did not inhibit the growth of gram-negative bacteria. Diethylstilbestrol was also found to inhibit the growth of *Lactobacillus helviticus* by Kodicek (1945). Reiss (1947) observed that testosterone inhibited the growth of two *Trichophyton* species and that estradiol had a small suppressive action on the same organisms. Diethylstilbestrol inhibited their growth only if the fungi were incubated with the steroid for two hours prior to their inoculation for the growth studies. The effects of progesterone on various microbes were studied by Casas-Campillo, *et al*. (1961). They found that progesterone inhibited the growth of a variety of pathogenic fungi, and that it also inhibited the growth of *Bacillus subtilis*, *S. aureus*, and some mycobacterium species, but had no effect upon *Candida albicans* and the gram-negative bacteria studied. Yotis and Stanke (1966) reported that the growth of four gram positive bacteria and of *C. albicans* was suppressed by progesterone. Yotis (1967) found that norethindrone suppressed the growth of *S. aureus*, *S. epidermidis*, *Micrococcus conglomeratus*, *Streptococcus faecalis*, and *Listeria monocytogenes*.

He also reported that when norethindrone and mestranol were combined, inhibition of the growth of S. aureus was greater than when either hormone was used alone. Baman (1968) found that seven species of gram-positive bacteria and also Saccharomyces cerevisiae were affected in their growth by diethylstilbestrol.

Most of these in vitro investigations have used a single steroid at concentrations higher than those found in normal human plasma. However, Yotis and Fitzgerald (1974) found that when plasma steroids were combined at their physiological concentrations, they inhibited the growth of S. aureus. This effect of the plasma steroids should be remembered when any attempt is made to correlate the in vitro effects of a single steroid with its in vivo effect, for a steroid does not act alone against an infectious agent in vivo but acts in conjunction with plasma steroids.

These and other studies clearly show that the in vitro growth of many gram-positive bacteria and of many fungi is suppressed by the sex steroids. Although the mechanism of steroid action on these microbes has not yet been firmly established, certain cell functions in addition to growth have been shown to be affected.

Schacter (1953) showed that the respiration of baker's yeast was affected by diethylstilbestrol when respiration was measured in a Warburg apparatus. He found that diethylstilbestrol concentrations below $1 \times 10^{-4}$ M and above $1 \times 10^{-3}$ M inhibited oxygen consumption by the yeast, but that concentrations between these values increased oxygen consumption by as mcuh as fourteen fold. Durham and Perry (1959) discovered in their manometric studies that diethylstilbestrol suppressed the endogeneous, but not

the exogeneous, respiration of Aerobacter aerogenes. Seven years later, Durham and Adams (1966) found that diethylstilbestrol inhibited the activity of partially purified A. aerogenes D-glucose-6-phosphate dehydrogenase. Diethylstilbestrol was shown by Yotis and Baman (1969) to also inhibit the exogeneous respiration of S. aureus.

The sex steroids have also been demonstrated to have an effect on the production of macromolecules by microbes. Jefferson (1967) showed that the production of yellow pigment by Aspergillis niger was increased by estradiol, estrone, progesterone, and diethylstilbestrol. Steroids were found to decrease the production of hemolysin by S. aureus in studies by Yotis and Yotis (1970).

Yotis and Fitzgerald (1969) discovered that the uptake of alanine by S. aureus was inhibited by sex steroids. Further studies by Yotis and Bhattacharyya (1971) showed that many of the sex steroids, including diethylstilbestrol, norethindrone, and mestranol, increased the release of alanine, lysine, and glutamic acid from the cellular pool of S. aureus into the environment. Fitzgerald and Yotis (1971) found that the entrance of alanine into different cellular fractions of S. aureus cells was retarded by progesterone. It was reported that when the concentration of progesterone increased, the number of fractions into which entrance was inhibited increased.

A recent experiment shows that sex steroids may bind preferentially to the mucopeptide portion of the bacterial cell wall. Yotis and Fitzgerald (1974) found that radio-labeled diethylstilbestrol, progesterone, and norethindrone, when added to different cellular fractions of S. aureus, bound preferentially to those fractions containing cell walls and mucopeptides.

The binding of sex steroids to the mucopeptide of cell walls and the effect of the steroids on permeability indicate that the effects of sex steroids may be associated with the cell envelope of bacteria. It would seem reasonable that if the steroid binds to the inner portion of the cell wall it could exert an effect on the proximal cytoplasmic membrane. The permeability effects of the hormone could account for the demonstrated effects on other microbial functions, since the cell would not operate efficiently under such conditions.

The mechanism of effect on the eukaryotic fungi could be the same; however, sex steroids have been shown to have intracytoplasmic effects in eukaryotic mammalian cells (Jensen and De Sombre, 1973). It is interesting to note that some experiments involving fungi and the sex steroids have had results dissimilar to that of the bacteria. Two such experiments are those cited above where sex steroids increased the production of pigment (Jefferson, 1967), and where diethylstilbestrol stimulated the respiration of yeast at certain concentrations (Schacter, 1953).

It is the purpose of this thesis to study some of the *in vitro* effects of diethylstilbestrol, norethindrone, and mestranol. Their effects on *C. albicans* and *S. pyogenes* are studied. Since many of the above studies have investigated the effects of steroids on *S. aureus*, an attempt is made to determine if similar effects occur on *C. albicans* and *S. pyogenes*. A study of the effects of the steroids found in plasma is included to determine if they nullify, enhance, or in any other way affect the actions of diethylstilbestrol, norethindrone, and mestranol.

## II. MATERIALS AND METHODS

Cultures. Candida albicans was obtained from Dr. T. Hashimoto, Loyola University of Chicago. Stock cultures were maintained on tryptic soy agar (Difco) slants at 4°C and were transferred to new slants every four weeks. Cultures were tested periodically for chlamydospore formation on corn meal agar (Difco) and for fermentation reactions in glucose, maltose, sucrose, and lactose.

Streptococcus pyogenes type 55, was obtained from Dr. C. F. Lange, Loyola University of Chicago. Stock cultures were maintained in Todd Hewitt Broth at 37°C and were transferred every few days. Cultures were tested periodically for gram-positive staining, catalase production, β-hemolysis on blood agar, and sensitivity to bacitracin.

Staphylococcus aureus serotypes IX, X and XII were used (ATCC 12606, 12607, 12609). Stock cultures were maintained on tryptic soy agar slants at 4°C and were transferred to new slants every four weeks. They were tested periodically for gram-positive staining, catalase production, pigment formation, glucose and mannitol fermentation, and coagulase production.

Steroids and steroid solutions. Powdered norethindrone and mestranol were obtained from Syntex Laboratories, Inc., Pala Alto, California; diethylstilbestrol dipropionate from Mann Research Laboratories, Inc., New York, New York; and diethylstilbestrol, androsterone, androsten-3b-17-diol, dehydroisoandrosterone, epiandrosterone, estrone, progesterone, cortisol, and cholesterol from Sigma Chemical Co., St. Louis, Mo. Fresh

solutions were prepared by dissolving a weighed amount (5-100 mg) of steroid in absolute ethanol. One part of this solution was then added to 99 parts of media or buffer to produce the desired steroid concentration in the solution. Controls received the same amount of ethanol.

Growth studies. Growth flasks were prepared by adding 1 part of the appropriate steroid solution or 1 part of absolute ethanol to 99 parts of growth medium. All media were then sterilized by autoclaving. Cells for the growth study were cultured at 37°C in similar media until the cells were in early stationary phase, harvested by centrifugation, and resuspended in sterile 0.85% saline. This suspension was then adjusted to the proper turbidity by means of a blue filter (#42) in a Klett-Summerson photoelectric colorimeter. Each flask of growth medium was then inoculated with this cell suspension and placed on a rotary shaker at 37°C. To adjust the inoculum, saline was used as a blank, and to adjust the growth flasks, uninoculated media was used as a blank for the colorimeter.

Growth was followed by turbidity measurements using the colorimeter as above, unless otherwise specified, or by actual cell counts. To measure the colony forming units (CFU) per ml in cultures of C. albicans, 0.5 ml aliquots of culture were removed and serially diluted in sterile 0.85% saline. In the first dilution tube, the cells were shaken on a Vortex Junior shaker for 30 seconds with about 15 glass beads with a diameter of 3 mm. A 0.1 ml aliquot of the desired dilution was spread on an agar plate, and a colony count was determined 24-48 hr later.

To measure the viable count, as CFU per ml, in cultures of S. pyogenes, 25 ml portions of the culture were removed and sonicated using a Branson

Instruments, Inc., Sonifier. The sonicator was adjusted to draw 2 amperes of current, and there was no cooling of the sample. After sonication, 0.5 ml samples of the culture were serially diluted in 0.85% saline. A 0.1 ml aliquot of the proper dilution was spread on each of three agar plates, and an average colony count was determined 24 hr later.

The number of cells in a culture of C. albicans were determined electronically with a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah,Florida). The appropriate threshold and current settings were determined by counting the number of cells in various standardized suspensions and systematically adjusting the threshold and current settings. The following settings were found to eliminate most background and yet give the highest cell counts: lower threshold, 15, upper threshold, 90, aperture current, 1/2, and amplification factor, 2. A correction factor for coincident counts was determined, but was insignificant at the level of accuracy needed for the growth curves. To count the cells in a culture, 5.0 ml samples were removed at the specified time and shaken on a Vortex Junior Shaker for 30 seconds with about 15 glass beads to break clumps of cells. The counts were then directly determined or were determined after serially diluting the sample in sterile tryptic soy broth to adjust the counts per ml to within the range of the instrument.

Manometric studies. Cells for the Warburg experiments were grown in 100 ml of broth, harvested by centrifugation, washed twice with saline, and adjusted to the appropriate turbidity in 0.1 M potassium phosphate buffer (pH 7.0). The suspension was divided into at least two portions, and one

part of hormone solution or ethanol was added to 49 parts of suspension. Incubation followed under varying conditions. A 1.0 ml sample was placed in the main compartment of a Warburg vessel along with 1.0 ml of buffered solution with the specified substrate. When glucose was used as a substrate, its final concentration was 5%. A 0.2 ml amount of 40% KOH and a filter paper wick were placed in the center well. Rates of oxygen uptake in air were determined by conventional manometric techniques at 37°C.

Leakage studies. Cells for leakage studies were grown in 100 ml of media, harvested by centrifugation, and resuspended in 0.1M potassium phosphate buffer (pH 7.0) to the desired turbidity. Uniformly labeled $^{14}$D-glucose ($2 \times 10^6$ counts/min/ml), obtained from Tracerlab, Waltham, Mass., was added to a final concentration of 1%. Cells were then incubated for 3 hr, harvested, washed twice in buffer, and resuspended in buffer to the original turbidity. The cell suspension was divided and steroid solution or ethanol added. Cells were incubated at 37°C on a shaker or in a Precision Thelco Anaerobic Incubator with a carbon dioxide atmosphere. To measure supernatant radioactivity 5 ml portions were removed, centrifuged at 10,000 x g for 10 minutes, and the supernatant recentrifuged. A 1.0 ml portion of each final supernatant was then added to a vial containing 10 ml of scintillation fluor. Alternatively, radioactivity of the cells was measured by filtering 1 ml of cell suspension with a 0.45 μm micropore filter, 13 mm in diameter. Filters were rinsed with 5 ml of distilled water, dried, and placed in vials containing 10 ml of scintillation fluor. The same scintillation fluor was used for both methods and was com-

posed of 150 ml of Anisole, 150 ml of 1, 2-dimethoxyethanol, 18 g of 2, 5-diphenyloxazole, and 60 mg of 1,4-bis 2-(5-phenyloxazolyl)-benzene dissolved in 900 ml of p-dioxane. $^{14}C$ activity of either the supernatant or the cell sample was determined in a Packard Tri-Carb scintillation spectrometer, Model 3320, which measured activity with an efficienty of 85%.

Cells used to study the loss of label from various cell fractions were treated as above until after the incubation with the steroid or ethanol. Then 15 ml samples of cell suspensions were harvested and washed twice in cold saline. At least two samples were fractionated by the method of Park and Hancock (1960). A 0.1 ml sample of each fraction was added to scintillation fluor. At least one 15 ml sample was used to count total cell activity by filtering a 1 ml sample and placing the filter in fluor. The activity of each sample was determined as in the leakage studies.

Labeled diethylstilbestrol studies. $^{14}C$ labeled DS (27.3 mci/m mol) was obtained from Nuclear Chicago, Chicago, Ill. The appropriate amounts of both labeled and unlabeled DS were dissolved in absolute ethanol to achieve a total concentration of 0.67 mg of DS per ml of ethanol and an activity of about 500,000 counts/min/ml. Growth flasks were prepared and inoculated as described under "Growth studies", except that the above steroid solution was used. The final concentration of label was 5,000 counts/min/ml, and the final concentration of DS was 6.7 µg per ml.

To obtain the background counts, 10 ml of autoclaved media was filtered through a 0.45 µm micropore filter. The filter was washed with 20 ml of 0.1 M potassium phosphate buffer (pH 7.0), dried, dissolved in scintillation fluor, and counted as in the labeled glucose studies. After the growth

flasks were inoculated, the activity of the cells was measured by similarly filtering a 10 ml sample of the culture, washing the filter, and adding the filter to fluor.

## III. RESULTS

### A. Effects of ethanol on the microbes studied.

Since it was necessary to add the steroids to the media as an ethanol solution, a similar amount of ethanol without added steroids was used as a control in all of the subsequent growth studies. The effects of 1% ethanol on growth are shown in Tables 1-3. The growth of Candida albicans and Staphylococcus aureus was affected, but that of Streptococcus pyogenes was not. The effect on C. albicans and S. aureus was not however, large. In several of the subsequent growth studies, a control was included which contained only media and no ethanol. The data from this control is not shown, but in every instance 1% ethanol had approximately the same effect as shown in Tables 1-3.

TABLE 1

Effect of ethanol on the growth of *C. albicans* [a]

| Trial | Additions | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 13 | 15 | 17 | 18 | 20 |
| 1 | None | 0 | 50 | 130 | 235 | 265 | 305 |
| | Ethanol, 1% | 0 | 25 | 85 | 190 | 235 | 310 |
| 2 | None | 0 | 25 | 85 | 195 | 255 | 330 |
| | Ethanol, 1% | 0 | 25 | 60 | 160 | 245 | 370 |
| 3 | None | 0 | 35 | 115 | 230 | 290 | 340 |
| | Ethanol, 1% | 0 | 30 | 80 | 175 | 255 | 340 |
| Mean | None | 0 | 37 | 110 | 220 | 270 | 325 |
| | Ethanol, 1% | 0 | 27 | 75 | 175 | 245 | 340 |

[a] A 0.5 ml amount of ethanol was added to 49.5 ml of tryptic soy broth in a nephelometric flask. The control flask contained only 50 ml of TSB. After sterilization by autoclaving, each flask was inoculated with about $1 \times 10^5$ cells and placed on a rotary shaker at 37° C. Growth was followed turbidmetrically in a Klett-Summerson colorimeter. The experiment was repeated twice to obtain the values given for trials 2 and 3.

TABLE 2

Effect of ethanol on the growth of S. pyogenes [a]

| Trial | Additions | Klett readings at various hours | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 6 | 7 | 8 | 9 |
| 1 | None | 0 | 35 | 80 | 105 | 100 |
| | Ethanol, 1% | 0 | 40 | 80 | 100 | 105 |
| 2 | None | 0 | 25 | 65 | 95 | 100 |
| | Ethanol, 1% | 0 | 20 | 60 | 95 | 95 |
| 3 | None | 0 | 25 | 50 | 100 | 105 |
| | Ethanol, 1% | 0 | 20 | 50 | 95 | 100 |
| 4 | None | 0 | 25 | 50 | 90 | 100 |
| | Ethanol, 1% | 0 | 25 | 55 | 95 | 100 |
| 5 | None | 0 | 35 | 75 | 105 | 105 |
| | Ethanol, 1% | 0 | 35 | 80 | 105 | 105 |
| Mean | None | 0 | 29 | 64 | 99 | 102 |
| | Ethanol, 1% | 0 | 28 | 65 | 98 | 101 |

[a] Experimental conditions were the same as in Table 1, except that about 1 x $10^6$ viable streptococci were inoculated into Todd Hewitt Broth, and the red filter (#660) of the colorimeter was used.

TABLE 3

Effect of ethanol on the growth of S. aureus [a]

| | | Klett readings at various hours | | | | |
|---|---|---|---|---|---|---|
| Trial | Additions | 0 | 11 | 12 | 13 | 14 |
| 1 | None | 0 | 215 | 325 | 375 | 415 |
| | Ethanol, 1% | 0 | 100 | 205 | 315 | 365 |
| 2 | None | 0 | 245 | 325 | 385 | 425 |
| | Ethanol, 1% | 0 | 205 | 305 | 380 | 420 |
| 3 | None | 0 | 145 | 215 | 245 | 275 |
| | Ethanol, 1% | 0 | 120 | 200 | 230 | 260 |
| 4 | None | 0 | 190 | 250 | 270 | 310 |
| | Ethanol, 1% | 0 | 135 | 200 | 240 | 270 |
| 5 | None | 0 | 105 | 185 | 290 | 285 |
| | Ethanol, 1% | 0 | 100 | 180 | 275 | 280 |
| 6 | None | 0 | 105 | 195 | 285 | 285 |
| | Ethanol | 0 | 95 | 180 | 275 | 285 |

[a] Experimental conditions were the same as in Table 1, except that about 1 x $10^6$ staphylococci were inoculated into Casein Hydrolysate Broth (BBL). Trials 1 and 2 were performed using Serotype IX, trials 3 and 4 using Serotype X, and trials 5 and 6 using Serotype XII.

B. Effects of Norethindrone and Mestranol.

Growth studies. The effects of norethindrone (NED) and norethindrone plus mestranol (NED-MS) were studied turbidimetrically as described. The results are shown in Tables 4-7. The data indicate that while NED and MED-MS may have some suppressive effects on the growth of C. albicans and S. pyogenes, their effects are not large when pharmacological concentrations (concentrations similar to those in the plasma of patients receiving NED-MS) are used.

Three separate experiments were performed with C. albicans to measure the effect of NED alone, and the results are shown in Table 4. Significant inhibition was observed only in early readings and particularly only when 15 µg of NED per ml were present. Under the same conditions for growth, NED was then combined with mestranol (MS). Since NED and MS are combined commercially in a ratio of approximately 10 parts of NED to 1 part of MS and sold commercially under the trademark "Norinyl", approximately the same ratio was used in these experiments. Table 5 shows the effects of NED-MS on the growth of C. albicans. The readings shown reveal that concentrations of 10 µg of NED and 1 µg of MS per ml caused slight suppression of growth.

The effects of NED on the growth of S. pyogenes were studied and the results are shown in Table 6. The results given and also the results of some preliminary studies indicate that NED had no effect on the growth of S. pyogenes in Todd-Hewitt Broth when concentrations of 5 to 20 µg of NED per ml were present. As with C. albicans, NED was also combined with MS, and the combined effects of the steroids were studied. As evident from

TABLE 4

Effect of norethindrone on C. albicans [a]

| Trial | NED µg/ml | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 14 | 16 | 18 | 20 | 22 |
| 1 | 0 | 0 | 70 | 160 | 325 | 375 | 395 |
| | 5 | 0 | 60 | 145 | 295 | 340 | 370 |
| | 10 | 0 | 60 | 140 | 290 | 340 | 365 |
| | 15 | 0 | 45 | 115 | 270 | 325 | 355 |
| 2 | 0 | 0 | 85 | 160 | 280 | 325 | 365 |
| | 5 | 0 | 75 | 150 | 265 | 310 | 355 |
| | 10 | 0 | 75 | 160 | 270 | 320 | 355 |
| | 15 | 0 | 70 | 140 | 245 | 315 | 345 |
| 3 | 0 | 0 | 85 | 165 | 270 | 330 | 380 |
| | 5 | 0 | 75 | 165 | 265 | 330 | 370 |
| | 10 | 0 | 70 | 155 | 250 | 320 | 355 |
| | 15 | 0 | 65 | 150 | 250 | 330 | 360 |
| Mean | 0 | 0 | 80 | 162 | 292 | 343 | 380 |
| | 5 | 0 | 70 | 153 | 275 | 327 | 365 |
| | 10 | 0 | 68 | 151 | 270 | 327 | 358 |
| | 15 | 0 | 60 | 135 | 255 | 323 | 353 |

[a] The appropriate amounts of dry steroid were dissolved in absolute ethanol, and then 0.5 ml of these solutions were added to 49.5 ml of tryptic soy broth in nephelometric flasks. The control flask received 0.5 ml of ethanol. After sterilization by autoclaving, each flask was inoculated with about $1 \times 10^5$ cells and placed on a rotary shaker at 37° C. Growth was followed turbidimetrically in a Klett-Summerson colorimeter. The experiment was repeated twice to obtain the values given for trials 2 and 3.

TABLE 5

Effect of NED and MS on C. albicans [a]

| Trial | NED µg/ml | MS µg/ml | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | 14 | 16 | 18 | 20 | 22 |
| 1 | 0 | 0 | 0 | 35 | 85 | 215 | 315 | 355 |
| | 10 | 1 | 0 | 30 | 70 | 190 | 280 | 330 |
| 2 | 0 | 0 | 0 | 40 | 80 | 205 | 320 | 365 |
| | 10 | 1 | 0 | 15 | 45 | 135 | 245 | 315 |
| 3 | 0 | 0 | 0 | 20 | 55 | 130 | 265 | 350 |
| | 10 | 1 | 0 | 15 | 45 | 95 | 240 | 330 |
| 4 | 0 | 0 | 0 | 40 | 95 | 205 | 270 | 324 |
| | 10 | 1 | 0 | 40 | 85 | 195 | 255 | 310 |
| Mean | 0 | 0 | 0 | 34 | 78 | 189 | 293 | 349 |
| | 10 | 1 | 0 | 25 | 61 | 154 | 255 | 321 |

[a] Experimental conditions were the same as in Table 4.

TABLE 6

Effect of NED on S. pyogenes [a]

| Trial | NED µg/ml | Klett readings at various hours | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 6 | 7 | 8 | 9 |
| 1 | 0 | 0 | 25 | 60 | 90 | 95 |
| | 5 | 0 | 25 | 55 | 85 | 90 |
| | 10 | 0 | 20 | 55 | 90 | 95 |
| | 20 | 0 | 25 | 50 | 90 | 100 |
| 2 | 0 | 0 | 15 | 35 | 70 | 100 |
| | 5 | 0 | 15 | 40 | 80 | 100 |
| | 10 | 0 | 15 | 40 | 80 | 100 |
| | 20 | 0 | 20 | 45 | 80 | 100 |
| Mean | 0 | 0 | 20 | 48 | 80 | 98 |
| | 5 | 0 | 20 | 48 | 83 | 95 |
| | 10 | 0 | 18 | 48 | 85 | 98 |
| | 20 | 0 | 23 | 48 | 85 | 100 |

[a] Experimental conditions were the same as in Table 4, except that about $1 \times 10^6$ streptococci were inoculated into Todd Hewitt Broth. Also, the red filter (#660) of the colorimeter was used.

TABLE 7

Effect of NED and MS on S. pyogenes [a]

| Trial | NED µg/ml | MS µg/ml | Klett readings at various hours | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 | 6 | 7 | 8 | 9 |
| 1 | 0 | 0 | 0 | 40 | 80 | 100 | 105 |
| | 10 | 1 | 0 | 30 | 60 | 95 | 90 |
| 2 | 0 | 0 | 0 | 20 | 60 | 95 | 95 |
| | 10 | 1 | 0 | 10 | 35 | 70 | 90 |
| 3 | 0 | 0 | 0 | 20 | 50 | 95 | 100 |
| | 10 | 1 | 0 | 15 | 30 | 70 | 95 |
| 4 | 0 | 0 | 0 | 25 | 55 | 95 | 100 |
| | 10 | 1 | 0 | 15 | 35 | 70 | 100 |
| 5 | 0 | 0 | 0 | 35 | 80 | 105 | 105 |
| | 10 | 1 | 0 | 20 | 40 | 80 | 100 |
| 6 | 0 | 0 | 0 | 20 | 45 | 85 | 100 |
| | 10 | 1 | 0 | 10 | 20 | 70 | 90 |
| Mean | 0 | 0 | 0 | 27 | 62 | 96 | 101 |
| | 10 | 1 | 0 | 17 | 37 | 76 | 94 |

[a] Experimental conditions were the same as in Table 6.

Table 7, NED-MS caused some suppression of the growth of S. pyogenes. The values tabulated are the results of six separate trials, and in all of these trials some suppression of growth occurred.

The suppressive effects of NED and NED-MS on the growth of C. albicans and S. pyogenes were considerably less than those of comparable concentrations of diethylstilbestrol and diethylstilbestrol dipropionate (see below). The effects of steroids on cellular functions other than the growth of S. pyogenes and C. albicans was, therefore, studied with diethylstilbestrol and diethylstilbestrol dipropionate rather than with NED-MS.

C. Effects of DS dipropionate and DS.

Growth studies. The effects of diethylstilbestrol (DS) and DS dipropionate on the growth of S. pyogenes and C. albicans were studied turbidimetrically and by viable counts (as colony forming units: CFU). The results shown in Tables 8-10, and in Fig. 1 and 3, show that DS dipropionate in concentrations of 5-20 µg per ml suppressed the growth of C. albicans, DS in concentrations of 7.5 and 10 µg per ml suppressed the growth of C. albicans, and DS in concentrations of 1 µg to 5 µg per ml suppressed the growth of S. pyogenes.

The effects of varying concentrations of DS dipropionate on the growth of C. albicans was assayed turbidimetrically, and the results are shown in Table 8. Growth was suppressed according to the amount of steroid present, and the suppression of growth decreased as the cells reached the stationary phase of growth. A concentration of 20 µg of DS dipropionate per ml was almost entirely inhibitory to growth.

When growth was studied by CFU as shown in Fig. 1, it became evident that the growth suppression caused by 15 µg of DS dipropionate per ml occurred only after 12 hr of growth. Examination of the curve thus obtained revealed that considerable growth had occurred before growth could be measured turbidimetrically at 15 hr (Table 8). Turbidity studies, therefore, did not show the point in growth at which suppression occurred.

Numerous studies performed only in segments of the growth period (0-9 hr, 12-21 hr, and 24-36 hr) confirm all aspects of the curves shown. The lag phase was no more than 3 hr. Growth was not affected by the steroid until after about 12 hr of growth, at which time it caused an abrupt slowing

TABLE 8

Effect of DS dipropionate (DS di) on C. albicans [a]

| Trial | DS di µg/ml | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 15 | 17 | 19 | 21 | 23 |
| 1 | 0 | 0 | 125 | 230 | 325 | 335 | 360 |
| | 10 | 0 | 15 | 45 | 80 | 125 | 190 |
| | 15 | 0 | 15 | 45 | 75 | 115 | 170 |
| | 20 | 0 | 10 | 45 | 75 | 120 | 180 |
| 2 | 0 | 0 | 95 | 230 | 295 | 315 | 340 |
| | 10 | 0 | 20 | 65 | 90 | 145 | 215 |
| | 15 | 0 | 10 | 55 | 75 | 125 | 185 |
| | 20 | 0 | 0 | 50 | 65 | 100 | 175 |
| 3 | 0 | 0 | 80 | 150 | 230 | 265 | 290 |
| | 10 | 0 | 40 | 70 | 115 | 165 | 230 |
| | 15 | 0 | 20 | 35 | 45 | 55 | 80 |
| | 20 | 0 | 0 | 20 | 20 | 25 | 30 |
| Mean | 0 | 0 | 100 | 203 | 283 | 305 | 330 |
| | 10 | 0 | 25 | 60 | 95 | 145 | 212 |
| | 15 | 0 | 15 | 45 | 65 | 95 | 145 |
| | 20 | 0 | 8 | 38 | 53 | 82 | 128 |

[a] Experimental conditions were the same as in Table 4.

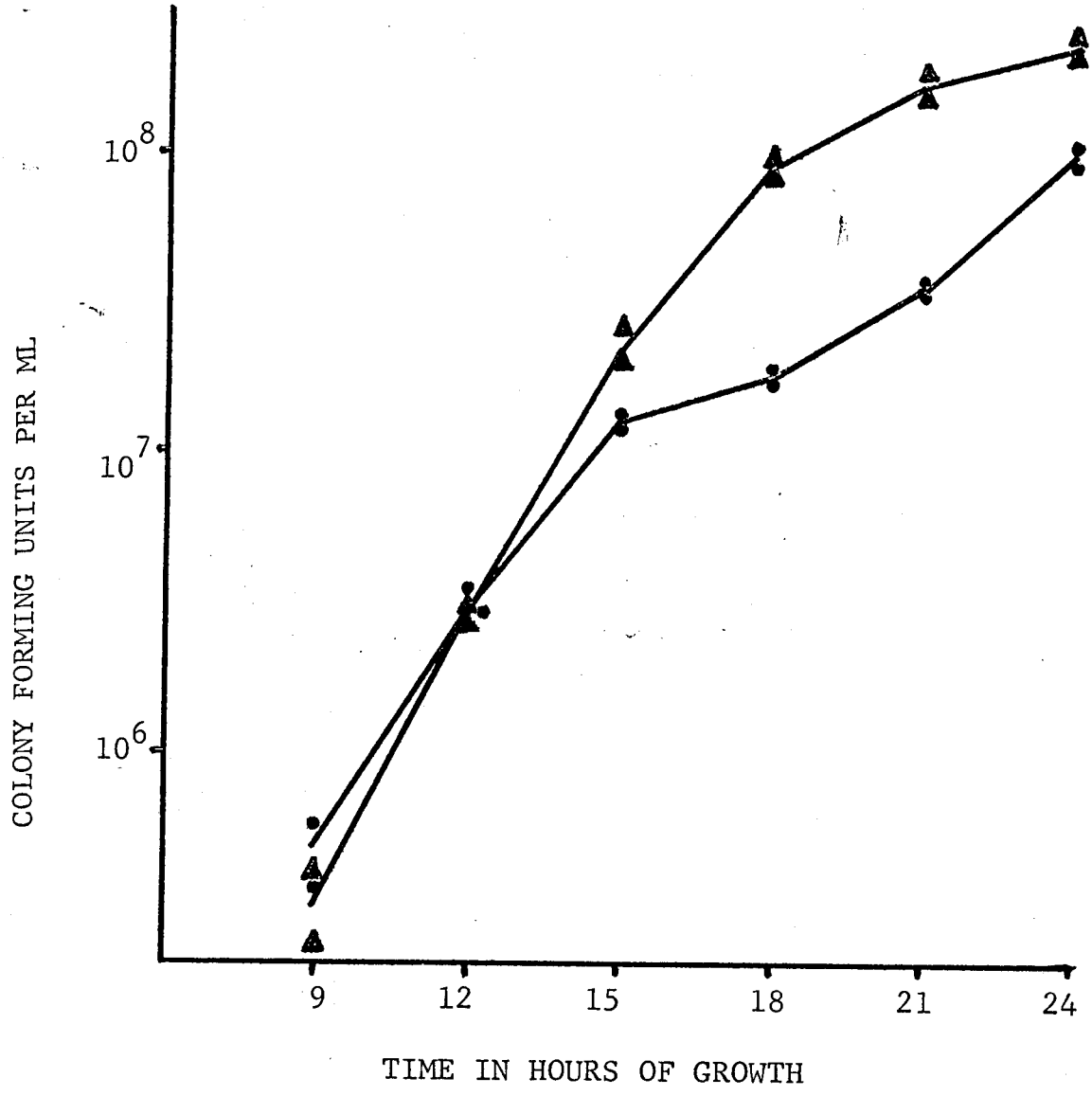



Fig. 1. Effect of DS dipropionate on the growth of C. albicans. Experimental conditions were the same as in Table 4, except that growth was determined by colony forming units. Each flask started growth with $1.7 \times 10^3$ CFU per ml of culture. The graph is drawn through the mean values for each hour. Control (▲); 15 μg of DS dipropionate per ml of media (●).

of late logarithmic growth. The effect slowly diminished until the viable counts of both the 1% ethanol control and the steroid containing culture were $2 \times 10^8$ CFU per ml.

The data in Table 9 compares the effects of DS and DS dipropionate on C. albicans. Complete inhibition of turbidity occurred when only 10 µg of DS per ml were present. In all other respects the inhibition caused by DS was comparable to that caused by DS dipropionate. Increased concentrations of DS caused increased suppression of growth, and suppression of growth decreased with the age of the culture.

The effects of varying concentrations of DS on the growth of S. pyogenes was also assayed turbidimetrically, and the results of three experiments are shown in Table 10. As with C. albicans, growth suppression was related to the amount of steroid present and decreased with time. A concentration of 5 µg of DS per ml entirely inhibited growth in all three trials for 10 hr, and after 22 hr, growth was evident in only two trials. Cells growing in 2.5 µg per ml had not yet fully recovered after 22 hr of growth, and 1.0 µg per ml caused significant suppression through 10 hr of growth.

The effect of 2.5 µg of DS per ml on the growth of S. pyogenes was studied by viable counts. Since the cell counts might have been affected by the tendency of streptococci to form chains, the possibility of sonicating the cells prior to dilution was explored. The effectiveness of sonication was studied in three separate trials, and each result is shown in Fig. 2. In consideration of these data, all cultures were sonicated for 1.5 min prior to serial dilution. The resultant growth curves are

TABLE 9

Comparative effects of DS and DS dipropionate (DS di) on *C. albicans* [a]

| Trial | Additions (μg/ml) | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 14.5 | 16.5 | 18 | 19 | 20.5 |
| 1 | 0 | 0 | 40 | 90 | 170 | 245 | 290 |
| | DS, 7.5 | 0 | 10 | 15 | 20 | 35 | 50 |
| | DS, 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | DS di, 10 | 0 | 5 | 30 | 60 | 90 | 155 |
| 2 | 0 | 0 | 20 | 55 | 120 | 170 | 245 |
| | DS, 7.5 | 0 | 0 | 0 | 0 | 0 | 5 |
| | DS, 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | DS di, 10 | 0 | 0 | 15 | 25 | 40 | 55 |
| 3 | 0 | 0 | 30 | 75 | 150 | 200 | 285 |
| | DS, 7.5 | 0 | 0 | 0 | 0 | 10 | 20 |
| | DS, 10 | 0 | 0 | 0 | 0 | 5 | 5 |
| | DS di, 10 | 0 | 10 | 25 | 45 | 55 | 95 |
| Mean | 0 | 0 | 30 | 73 | 147 | 205 | 273 |
| | DS, 7.5 | 0 | 3 | 5 | 7 | 15 | 25 |
| | DS, 10 | 0 | 0 | 0 | 0 | 2 | 2 |
| | DS di, 10 | 0 | 8 | 23 | 43 | 65 | 102 |

[a] Experimental conditions were the same as in Table 4.

TABLE 10

Effect of DS on S. pyogenes [a]

| Trial | DS µg/ml | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 6 | 7 | 8 | 10 | 22 |
| 1 | 0 | 0 | 35 | 80 | 105 | 105 | 90 |
| | 1.0 | 0 | 20 | 40 | 70 | 85 | 90 |
| | 2.5 | 0 | 0 | 5 | 10 | 20 | 85 |
| | 5.0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 2 | 0 | 0 | 20 | 60 | 95 | 95 | 95 |
| | 1.0 | 0 | 10 | 35 | 75 | 85 | 90 |
| | 2.5 | 0 | 0 | 0 | 5 | 15 | 75 |
| | 5.0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 20 | 50 | 95 | 100 | 110 |
| | 1.0 | 0 | 10 | 15 | 25 | 80 | 105 |
| | 2.5 | 0 | 0 | 0 | 0 | 0 | 80 |
| | 5.0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Mean | 0 | 0 | 27 | 63 | 97 | 98 | 102 |
| | 1.0 | 0 | 13 | 30 | 55 | 83 | 95 |
| | 2.5 | 0 | 0 | 0 | 0 | 12 | 80 |
| | 5.0 | 0 | 0 | 0 | 0 | 0 | 3 |

[a] Experimental conditions were the same as in Table 6.

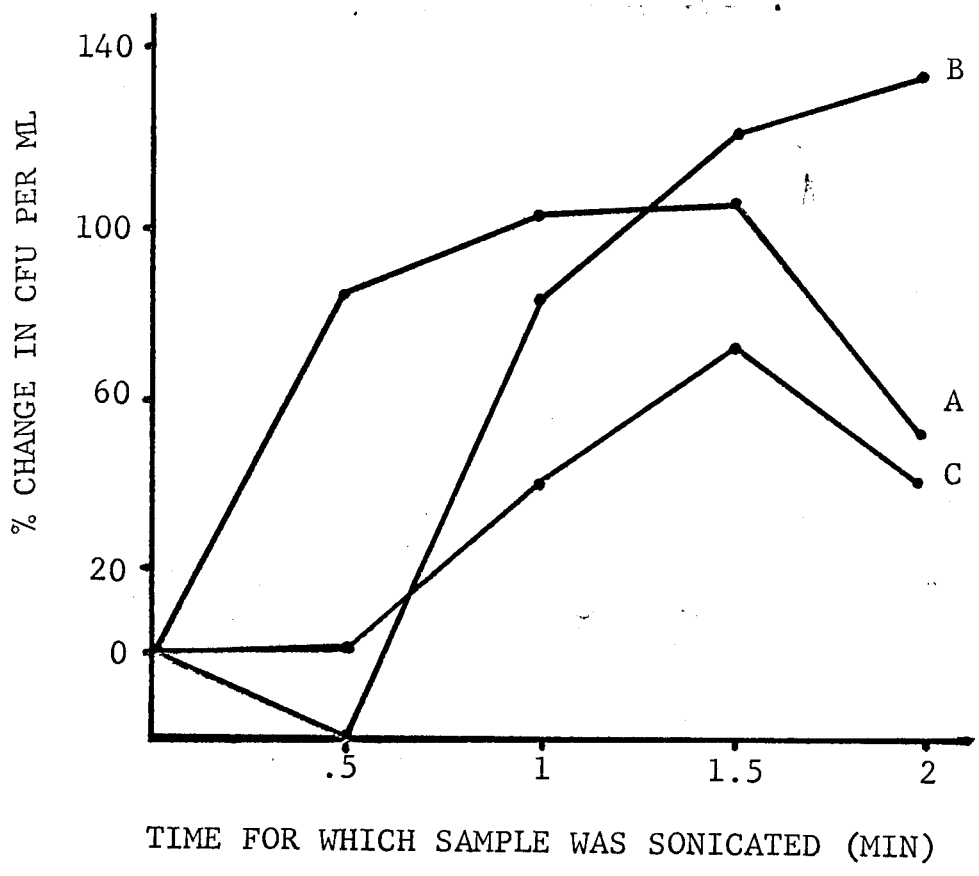



Fig. 2. Effect of sonication on S. pyogenes. Viable streptococci were inoculated into 3 flasks, containing either THB (A), 1% ethanol in THB (B), or 2.5 μg of DS per ml of THB (C). The flasks were incubated at 37°C for either 2 hr (A) or 6 hr (B and C). Flask A had $4 \times 10^7$ viable units/ml at 0 min, B had $8 \times 10^5$, and C had $1 \times 10^6$. Each culture was then divided into 4 or 5 25ml samples, and each sample was sonicated for the designated time. CFU were measured before and after sonication. Per cent change in CFU was calculated by the following formula:

$$\frac{\text{CFU after sonication - CFU before sonication}}{\text{CFU before sonication}} \times 100\%$$

shown in Fig. 3. DS had an immediate effect on growth, causing a much longer lag phase, and it caused a decreased rate of growth during the logarithmic phase.

Manometric studies. The effects of DS dipropionate and DS on the respiration of C. albicans and S. pyogenes, respectively, were studied with a Warburg apparatus as described in Materials and Methods (Tables 11-12).

Oxygen uptake studies were performed with C. albicans using 10 to 100 µg of DS dipropionate per ml under varying conditions of measurement. Cellular concentrations varied from 200 to 500 Klett units, and prior incubations with the steroid varied from 0 to 18.5 hr either at 4°C or 37°C. Under the various conditions, uptake of oxygen varied from 85 µl to 425 µl of oxygen per hour, but no significant differences were caused by the DS dipropionate. Typical results are given in Table 11.

Table 12 shows the results of manometric studies with S. pyogenes. Oxygen consumption was small and did not continue for more than about 2 hours, but 10 µg of DS per ml appeared to have a suppressive effect on the exogeneous respiration of Todd-Hewitt broth.

Labeled glucose studies. Since several steroids have been shown to increase the release of cellular constituents from S. aureus (Yotis and Bhattacharyya, 1971), the effects of DS and DS dipropionate on the leakage of cellular constituents from C. albicans and S. pyogenes were studied (Table 13).

Under the experimental conditions used, DS dipropionate did not affect the release of cellular constituents from C. albicans. The studies were performed as described and were exactly similar to those previously

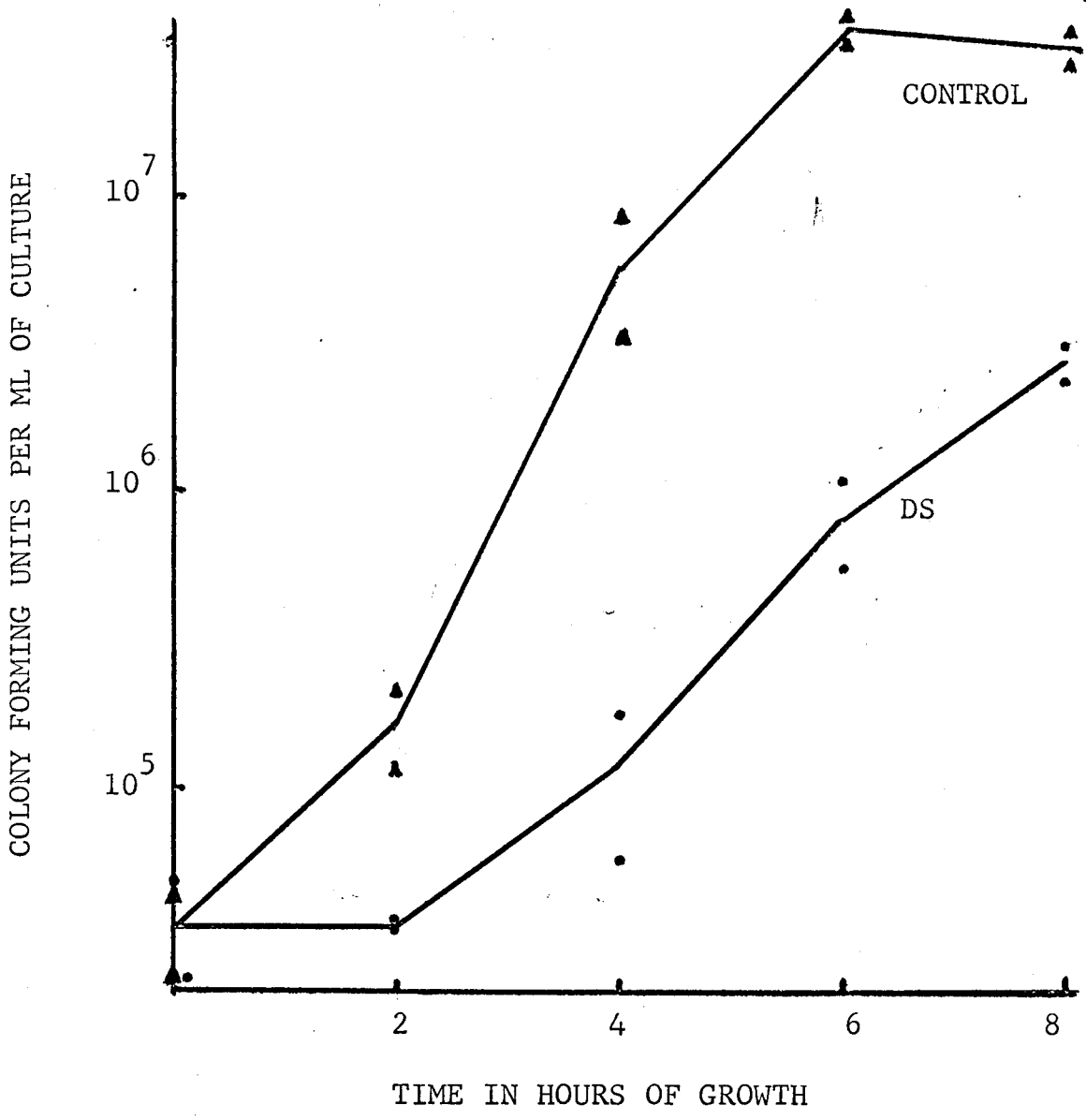



Fig. 3. Effect of DS on the growth of S. pyogenes. Approximately 7 x $10^6$ viable streptococci were inoculated into duplicate flasks containing 200 ml of THB with the designated additions. Colony forming units were determined after sonication of a 25 ml sample for 1.5 min as described in the legend for Fig. 2. The graph is drawn through the mean values for each hour. Control (▲); 2.5 μg of DS per ml of media (●).

TABLE 11

Effect of DS dipropionate on the respiration of *C. albicans* [a]

| Trial | Klett units | Incubation | DS di µg/ml | µg of $O_2$ consumed/hr | |
|---|---|---|---|---|---|
| | | | | 1st hr | 2nd hr |
| 1 | 500 | None | 0 | 425 | 366 |
| | | | 10 | 391 | 308 |
| 2 | 500 | None | 0 | 337 | 301 |
| | | | 10 | 342 | 293 |
| 3 | 200 | None | 0 | 115 | 108 |
| | | | 10 | 121 | 112 |
| 4 | 200 | 1.5 hr | 0 | 115 | 100 |
| | | | 20 | 122 | 98 |
| 5 | 200 | 18.5 hr | 0 | 86 | 94 |
| | | | 20 | 85 | 89 |
| 6 | 400 | 18.5 hr | 0 | 180 | 204 |
| | | | 100 | 190 | 209 |

[a] Cells were suspended in buffer to the specified turbidity, and steroid solution or ethanol (2%) was added. After incubation at 37°C for the specified time, the respiration of glucose was measured by conventional manometric techniques in a Warburg apparatus. Figures given are the mean of 2-3 flasks from the same trial.

TABLE 12

Effect of DS on the respiration of S. pyogenes [a]

| Trial | Additions (μg/ml) | Substrate | μl of $O_2$ consumed/hr 1 hr | 2nd hr |
|---|---|---|---|---|
| 1 | None | None | 0 | 0 |
| | None | THB[b] | 27 | 19 |
| | Ethanol (1%) | THB | 28 | 25 |
| | DS, 10 | THB | 18 | 12 |
| 2 | None | None | 4 | 0 |
| | None | THB | 24 | 19 |
| | Ethanol (1%) | THB | 24 | 15 |
| | DS, 10 | THB | 5 | 10 |
| 3 | None | None | 28 | 0 |
| | None | THB | 44 | 0 |
| | Ethanol (1%) | THB | 53 | 0 |
| | DS, 10 | THB | 36 | 0 |
| Mean | None | None | 11 | 0 |
| | None | THB | 32 | 13 |
| | Ethanol (1%) | THB | 35 | 13 |
| | DS, 10 | THB | 20 | 7 |

[a] Late logarithmic phase cells were suspended in buffer, and the specified additions were made. The respiration of the specified substrates was immediately measured by conventional manometric techniques in a Warburg apparatus. Values given are the mean of 2-3 flasks from the same trial.

[b] THB: Todd-Hewitt Broth

TABLE 13

Effect of DS dipropionate on the permeability of C. albicans.

| | CPM of cells after incubation with DSdi | | |
|---|---|---|---|
| | | DSdi | DSdi |
| Trial | Control | 25 µg/ml | 50 µg/ml |
| 1 | 14,000 | 12,000 | 14,000 |
| | 12,000 | 13,000 | 14,000 |
| 2 | 9,500 | 11,500 | 11,500 |
| | 9,000 | 12,000 | 9,500 |
| 3 | 10,500 | 11,500 | 11,500 |
| | 11,000 | 12,000 | 12,000 |
| Mean | 11,000 | 12,000 | 12,500 |

[a] A 100 Klett suspension of C. albicans was prepared and allowed to metabolize uniformly labeled $^{14}C$ glucose. Then, either steroid solution or ethanol (1%) was added, and the suspension was placed in an anaerobic incubator at 37°C for 18 hr. The suspensions were then filtered, and the filter was washed, dried, and dissolved in scintillation fluor. The CPM obtained and given above were each from separate test tubes incubated in the same trial.

performed on S. aureus by other researchers. DS dipropionate concentrations of 25 to 50 µg per ml of buffer were used. Cellular concentrations varied from 100 to 200 Klett units, and incubations with the steroid ranged from 2 to 18 hr, either aerobically or anaerobically. Radioactivity was measured in either the supernatant or the cells. No significant effect was found under any of these conditions (Table 13).

Fitzgerald (1971) found that steroids affect the incorporation of labeled glucose into certain cellular fractions of S. aureus. The same experiment was performed with C. albicans, using 10 µg of DS dipropionate per ml as shown in Table 14, but no significant effect was found.

Labeled glucose studies with S. pyogenes were very inconclusive. The uptake or the leakage of cellular constituents was not a stable occurrence, for values obtained from even the controls alone varied widely in the different trials. This might be expected, since Warburg studies indicated that cellular activity was low under other than optimum conditions.

TABLE 14

Fractionation studies [a]

| Trial | µgDSdi/ml | Counts per minute per ml of fraction | | | | | |
|---|---|---|---|---|---|---|---|
| | | Cold TCA soluble | Ethanol soluble | Hot TCA soluble | Trypsin degraded | Residue | Whole cells |
| 1 | 0 | 10,000 | 7,800 | 24,000 | 1,200 | 17,000 | 129,000 |
| | 10 | 11,000 | 11,000 | 22,500 | 1,000 | 16,000 | 114,000 |
| 2 | 0 | 9,000 | 6,500 | 21,000 | 1,000 | 16,000 | 69,000 |
| | 10 | 6,550 | 7,000 | 24,000 | 1,200 | 15,500 | 90,000 |

[a] A 100 Klett suspension of C. albicans was prepared and allowed to metabolize uniformly labeled $^{14}C$ glucose. Then either steroid solution or ethanol (1%) was added, and the suspension was placed in an anaerobic incubator at 37°C for 20 hr. Samples of 15 ml were fractionated by the method of Park and Hancock (1960). Aliquots of each fraction were added to scintillation fluor, and the counts/min obtained. Whole cells were filtered, and the counts/min of the filter was obtained. The amount of cells was such that the figures for whole cells above represents the sum of the fractions.

D. Further investigations of the effect of DS on C. albicans.

Growth studies. The delayed effect of DS dipropionate on the growth of C. albicans (Fig. 1) is quite different from the immediate effect of DS on S. pyogenes (Fig. 3) and S. aureus (Baman, 1968). It was, therefore, decided to further investigate this delay.

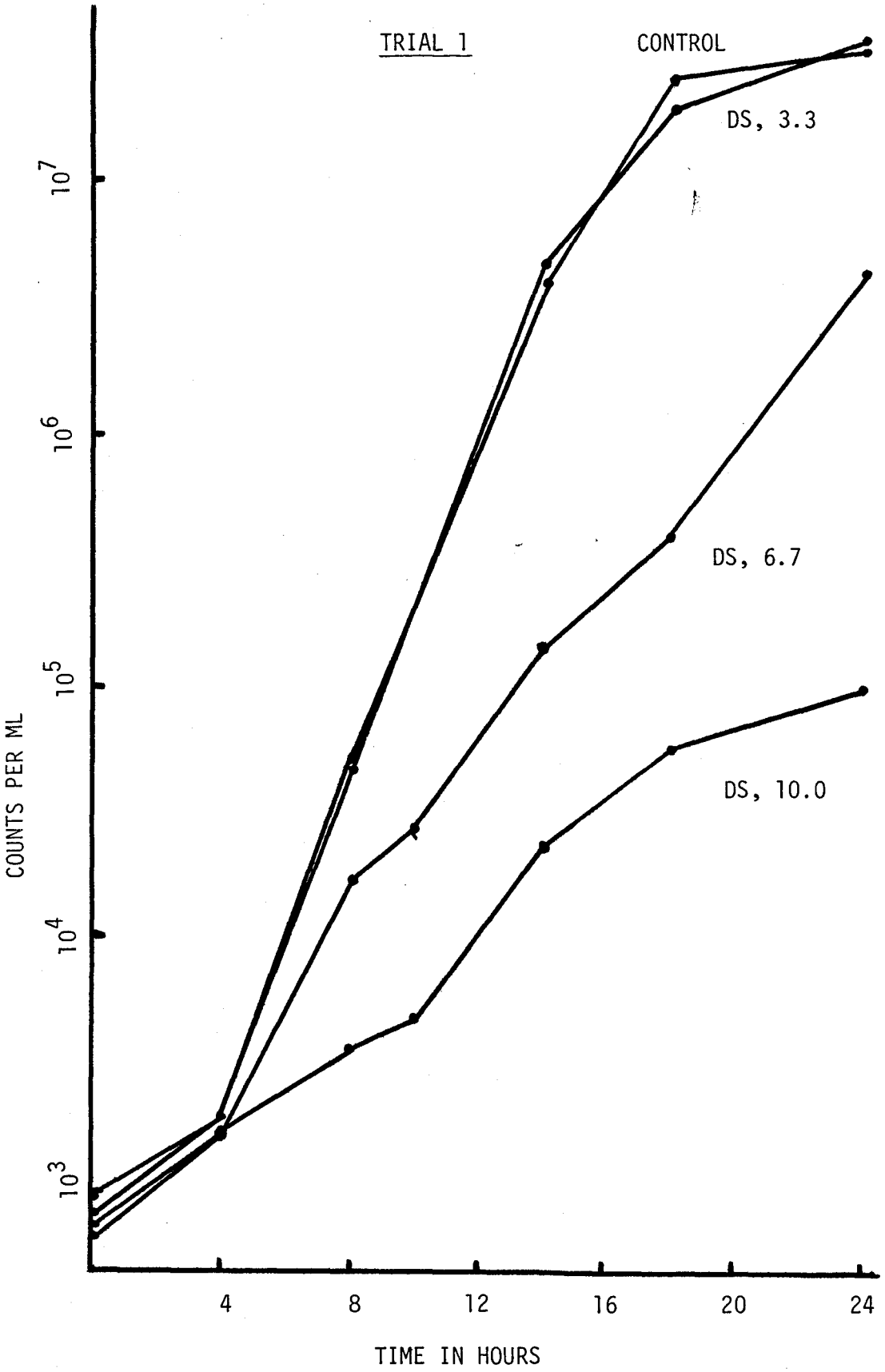
DS rather than DS dipropionate was used in these studies for several reasons, one of which was to determine whether a delayed effect was unique to the dipropionate form of diethylstilbestrol. The data show that 6.7 µg of DS per ml has a delayed effect in mid-logarithmic phase as does 15 µg of DS dipropionate per ml (Fig. 4).

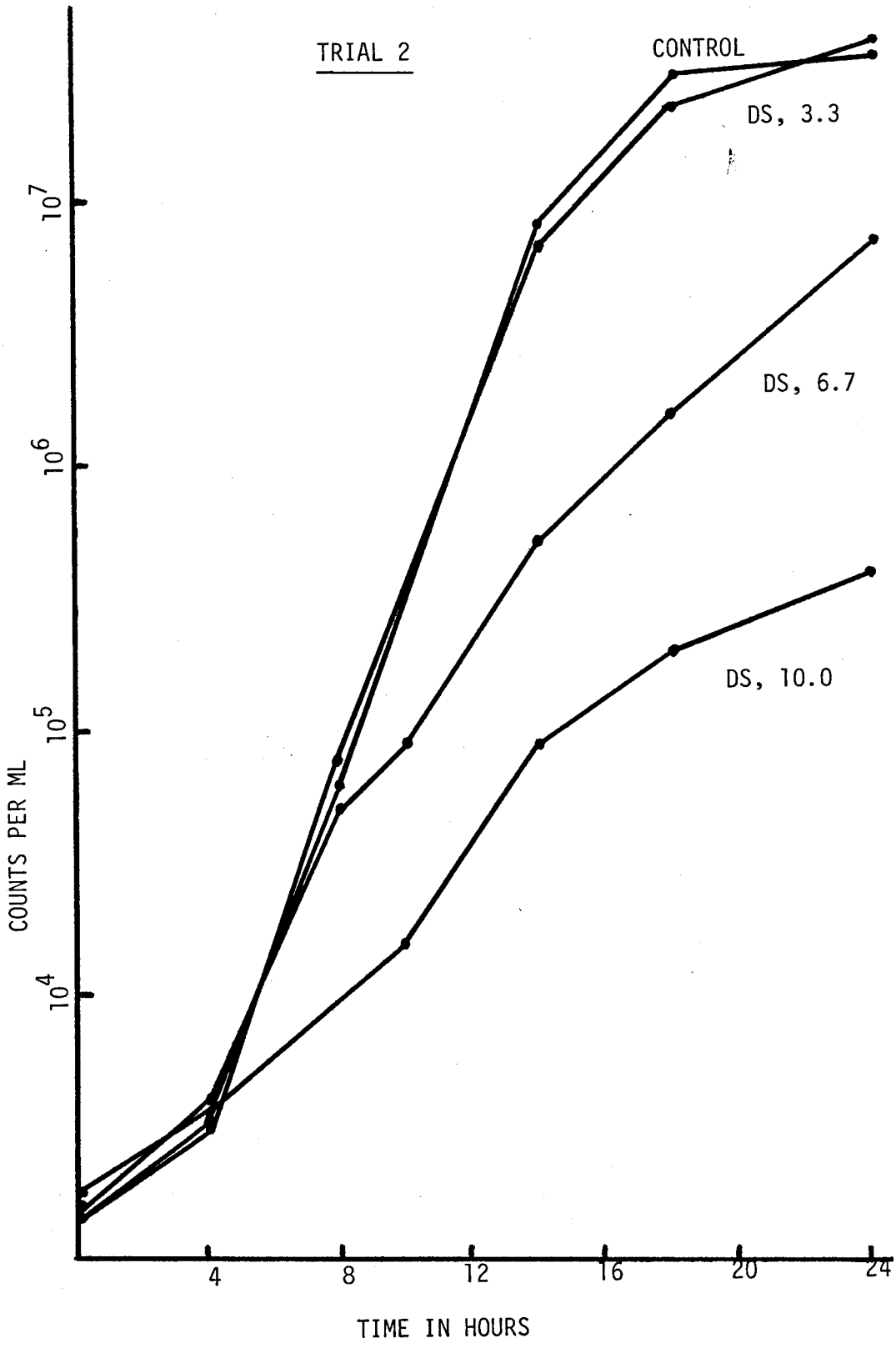
Fig. 4 also shows the effects of varying concentrations of DS. The data reveal that different concentrations of DS affect C. albicans at various stages of growth. The largest concentration used, 10 µg per ml, had an effect after only a little growth had occurred, but the smallest, 3.3 µg per ml, appeared to have a small effect almost at the point where the cells entered the stationary phase.

These results eliminate many changes in the media which might have caused a delay in effect. If the pH, oxygen tension, accumulated wastes, etc., had caused susceptibility to DS, these varying concentrations would probably have caused an effect at the same point in growth.

Some preliminary experiments (results not shown) indicated that when varying sizes of inoculum were used, 6.7 µg of DS caused an almost equal effect after similar amounts of growth. Based on this finding the possibility of using a much larger inoculum than that previously used was explored. The growth of cells could then be followed turbidimetrically,

Fig. 4. Effect of varying concentrations of DS on C. albicans. Cells were inoculated into duplicate flasks containing 150 ml of TSB with the designated additions and incubated on a rotary shaker at 37°C. At the appropriate times, two samples were removed from each culture to be counted by a Coulter Counter. When necessary, the samples were serially diluted in TSB to place the counts within the range of the instrument. Data for each of the following figures are the mean of two counts obtained in a single trial. Amounts of DS are given in μg per ml.


TRIAL 1
CONTROL
DS, 3.3
DS, 6.7
DS, 10.0
COUNTS PER ML
$10^3$
$10^4$
$10^5$
$10^6$
$10^7$
4
8
12
16
20
24
TIME IN HOURS


TRIAL 2
CONTROL
DS, 3.3
DS, 6.7
DS, 10.0
COUNTS PER ML
$10^4$
$10^5$
$10^6$
$10^7$
4
8
12
16
20
24
TIME IN HOURS

and the larger amounts of cells could be more easily studied. The upper portion of Table 15 shows that 6.7 µg of DS per ml caused a significant effect after 4-5 hr, when 50 ml of media were inoculated with about 5 x $10^7$ cells. The effect was seen earlier than when a smaller inoculum was used (Fig. 4), but the lag phase was considerably shorter. These data not only show that a larger inoculum can be used, but also confirm the conclusions from Fig. 4. If a change had occurred in the media which caused susceptibility, this change would probably have occurred much sooner with a large inoculum.

Since the growth of cells appeared to be an important factor in the absorption of DS, the absorption of DS by the cells during their growth was studied (Table 15).

To measure the absorption of DS, labeled DS was added to the media along with unlabeled DS for a total concentration of 6.7 µg of DS per ml. The media were autoclaved and inoculated in the usual manner for growth studies. At various time intervals the amount of label absorbed by the cells was measured by collecting cells on a filter and placing the filter in a scintillation vial for counting. Although little DS was absorbed by the cells, it was evident that significant amounts were not absorbed until after 4 hr in the media. It is also significant that during preliminary studies it was found that any more washing of the cells than that indicated in Table 15 caused the cells to lose all label. The DS was, therefore, loosely bound.

TABLE 15

Absorbtion of labeled DS by C. albicans [a]

| Trial | DS µg/ml | Klett readings at various hours | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | 0 | 9 | 10 | 17 | 24 | 37 | 64 | 110 |
| | 6.7 | 11 | 10 | 16 | 22 | 32 | 51 | 75 |
| 2 | 0 | 13 | 12 | 16 | 25 | 40 | 66 | 110 |
| | 6.7 | 11 | 11 | 16 | 25 | 36 | 56 | 76 |
| 3 | 0 | 11 | 11 | 18 | 24 | 39 | 65 | 109 |
| | 6.7 | 11 | 11 | 18 | 24 | 37 | 53 | 77 |
| Mean | 0 | 11 | 11 | 17 | 24 | 39 | 65 | 110 |
| | 6.7 | 11 | 11 | 17 | 24 | 35 | 53 | 76 |

| Trial | Blank | CPM x $10^3$ per 10 ml of cells at various hours | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | 2.3 | 2.4 | 2.9 | 2.4 | 2.4 | 3.8 | 5.0 | 3.3 |
| 2 | 2.1 | 2.0 | 2.3 | 2.6 | 2.5 | 3.9 | 4.9 | 2.9 |
| 3 | 1.8 | 2.1 | 2.6 | 2.5 | 1.9 | 3.0 | 3.6 | 3.2 |
| Mean | 2.1 | 2.2 | 2.6 | 2.5 | 2.3 | 3.6 | 4.5 | 3.1 |

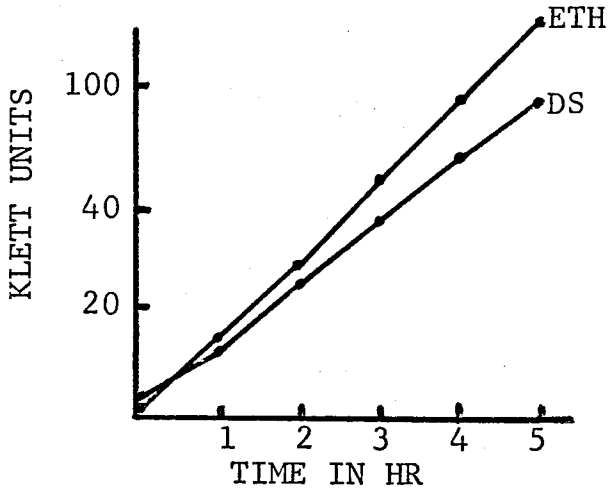
[a] C. albicans was inoculated into triplicate flasks of tryptic soy broth containing $C^{14}$ labeled DS, and growth was followed turbidimetrically. At the designated times, 10 ml of culture were removed and filtered. The filter was washed with 20 ml of 0.1 M phosphate buffer (pH 7.0), dried and dissolved in scintillation fluor to obtain the CPM.

It was still not apparent why the cells did not immediately absorb significant levels of DS. Since the data shown in Fig. 4 had eliminated many of the possibilities of changes in the media causing susceptibility to DS, the possibilities of changes in the cells were considered.

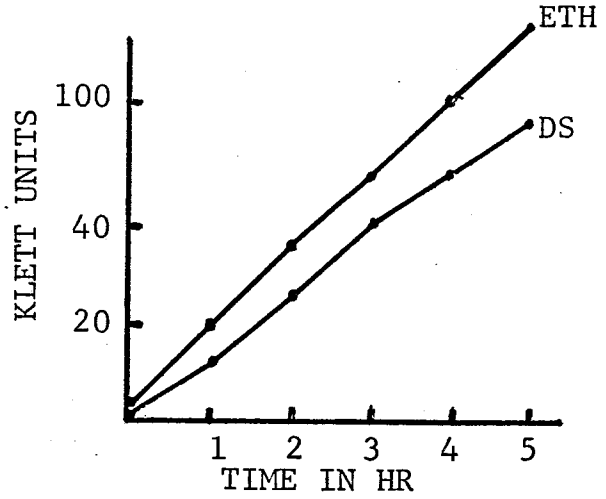
One of these hypotheses was that the age of the cells in the media determined susceptibility. To test this, cells were cultured for 4 hr in TSB under the same conditions used in the above growth study, and then the cells were collected and reinoculated into fresh TSB with 6.7 µg of DS per ml. Fig. 5 shows the results of this study. Cells cultured for 4 hr in TSB with or without additions were immediately susceptible to the DS. Cells treated similarly, but not cultured for 4 hr were not immediately susceptible.

This result explains not only the delayed effect of 6.7 µg of DS, but also the lack of influence of the state of the media (as seen in Fig. 4) and the delayed absorption of DS (Table 15). The reasons why growth in tryptic soy broth has this effect are, of course, not revealed by these data.

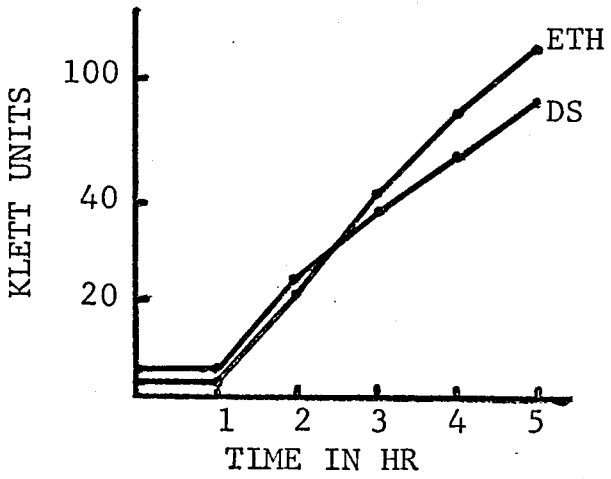
Fig. 5. Effect of prior growth in TSB on the effects of DS. C. albicans was inoculated into duplicate 2 liter flasks containing 1000 ml of TSB with the additions indicated under each figure. At the time indicated under each figure the cells were harvested, washed, and resuspended into saline. The cells from each 2 liter flask were then inoculated into 50 ml of TSB in a 250 ml nephelometric flask with the additions indicated inside each figure. Growth was then followed turbidimetrically. Three separate trials were performed, and the data from each are given separately. The concentration of DS used was 6.7 μg per ml of media.




NO ADDITIONS - FOR 4 HR

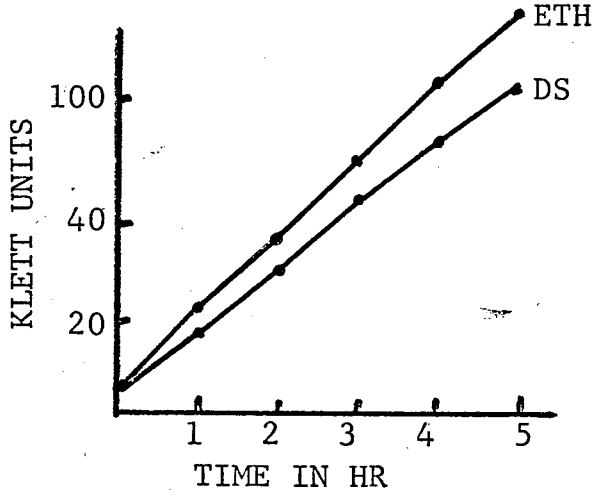



DS, 6.7 - FOR 4 HR

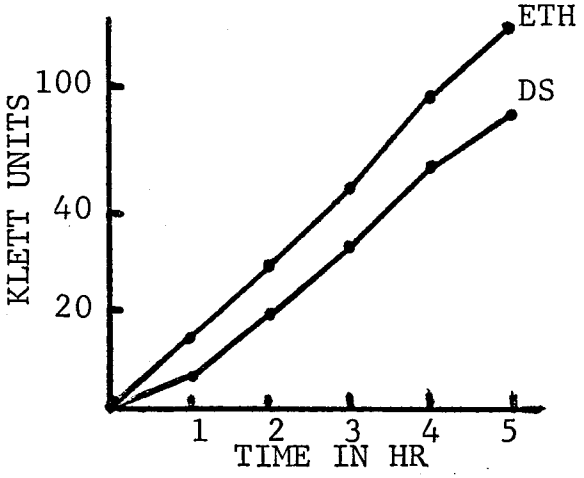



NO ADDITIONS - FOR 0 HR

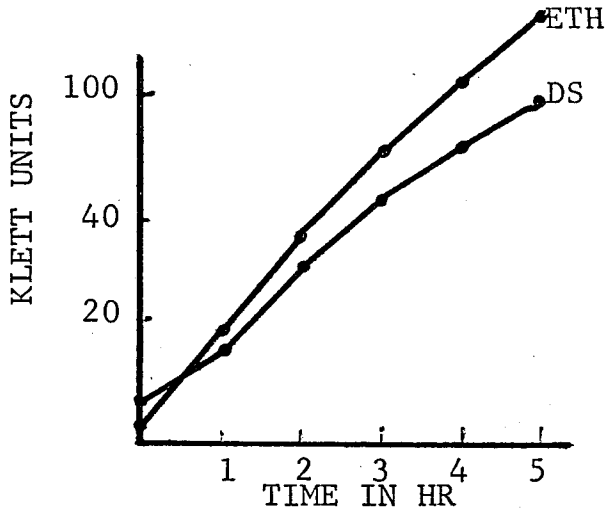
TRIAL 1



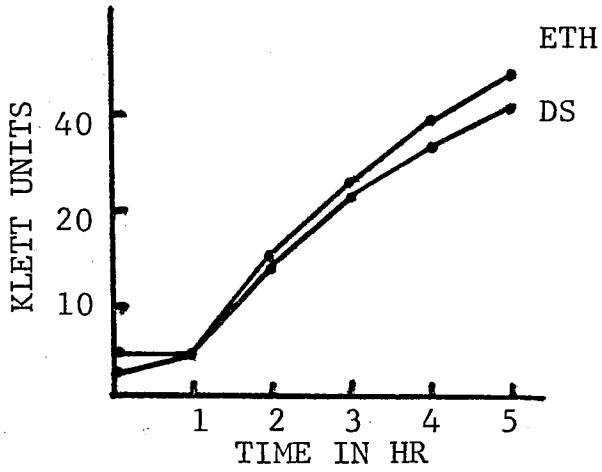

ETHANOL - FOR 4 HR




NO ADDITIONS - FOR 4 HR

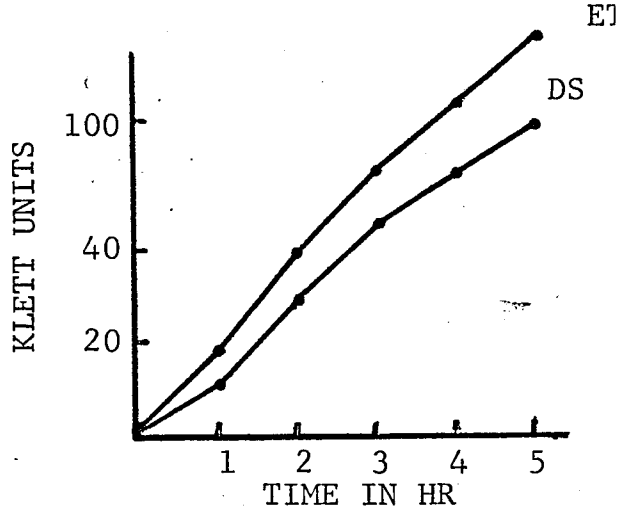



DS, 6.7 - FOR 4 HR

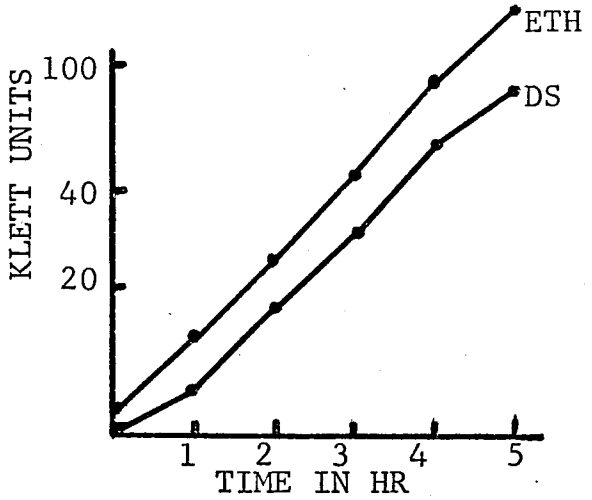



NO ADDITIONS - FOR 0 HR

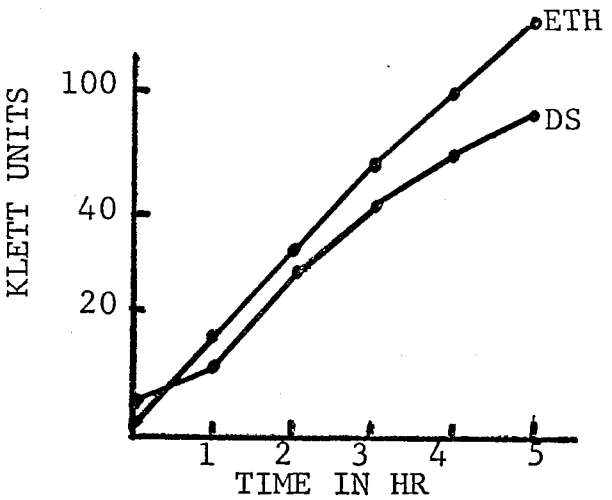
TRIAL 2



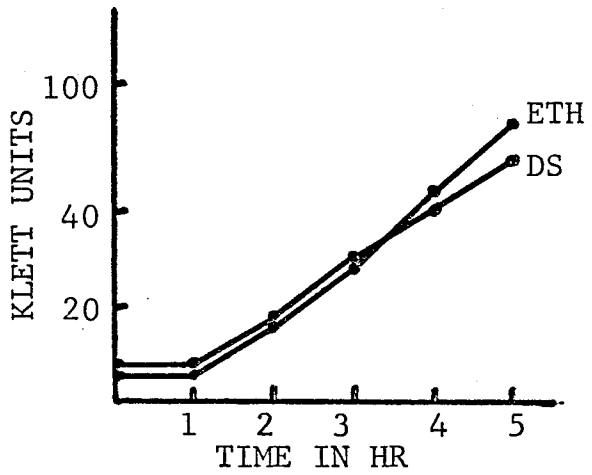

ETHANOL - FOR 4 HR




NO ADDITIONS - FOR 4 HR

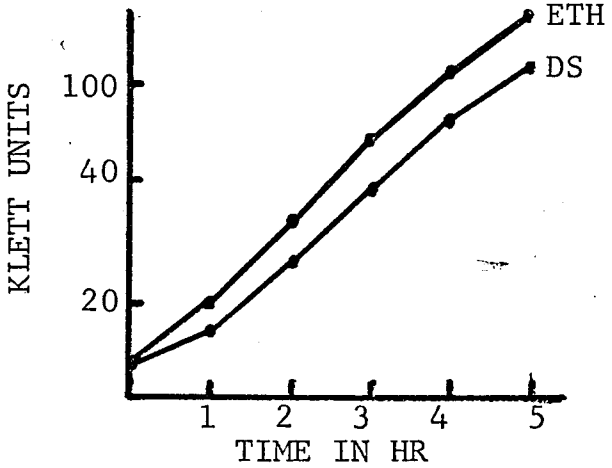



DS, 6.7 - FOR 4 HR




NO ADDITIONS - FOR 0 HR

TRIAL 3




ETHANOL - FOR 4 HR

E. Effects of DS and NED-MS in Conjunction with Plasma Steroids.

Growth studies. These and other studies (Yotis, 1967; Baman, 1968; Yotis and Baman, 1969), indicate that DS, DS dipropionate, NED, and MS have in vitro suppressive effects on the growth of some pathogenic microbes. In an in vivo situation, however, these pharmaceutical steroids would not act alone on microbes, but would act along with the steroids present in plasma. Since plasma steroids have been shown to have an in vitro suppressive effect on microbes (Yotis and Fitzgerald, 1974), the effects of DS, DS dipropionate, and NED-MS were studied in conjunction with a combination of steroids similar to that in normal plasma.

The concentrations of plasma steroids used in the study are shown in Table 16. The steroids used do not include all steroids in plasma, but do include many of those present in significant concentrations which have been shown separately to affect microbial growth. This same combination was shown by Yotis and Fitzgerald (1974) to suppress the growth of certain S. aureus serotypes. The plasma combination of steroids (PCS) was added to growth medium in their normal plasma concentrations by first dissolving weighed amounts of each steroid in ethanol, and then adding 1 part of this solution to 99 parts of media. If DS, DS dipropionate, or NED-MS were used in conjunction with the PCS, a weighed amount of this pharmaceutical steroid was added to the ethanol solution prior to addition to the media.

Table 17 shows that the PCS suppressed the growth of C. albicans, S. pyogenes, and three serotypes of S. aureus. In Tables 18-24, it is seen that even more inhibition of growth may occur when DS, DS dipropionate, or NED-MS is added to the PCS.

TABLE 16

Steroids used to create an *in vitro* combination similar to the physiological levels in normal human plasma [a]

| Steroid | Concentration in media (µg/ml) |
|---|---|
| Androsterone | 0.50 |
| Androsten-3b-17-diol | 0.25 |
| Dehydroisoandrosterone | 2.00 |
| Epiandrosterone | 0.10 |
| Estrone | 0.10 |
| Progesterone | 0.40 |
| Cortisol | 0.20 |
| Cholesterol | 10.00 |

[a] All steroids were weighed in milligram amounts and then dissolved together in absolute ethanol. One part of this solution was then added to 99 parts of growth media to give the final concentrations shown above. These final concentrations are approximately the same as those given for the same steroids in human plasma by Gray and Brown (1967) and Antonoides (1960). The value for cholesterol is, however, lower than that in plasma due to its low solubility in water and the subsequent impracticability of large amounts in our system.

The suppressive effects of the PCS varied widely with the organisms studied (Table 17). The greatest suppression occurred with S. pyogenes, where 100% inhibition occurred for 7 hr. Least suppression was present with S. aureus Serotype XII, where the PCS caused only 8-10% inhibition. The other serotypes of S. aureus and C. albicans were suppressed from 17-62% at various hours. As in previous studies dealing with the effects of steroids on microbial growth, suppression was greatest at early readings and decreased with time.

Table 18 shows the effect of DS dipropionate on C. albicans in conjunction with the PCS, and Table 19 shows the effect of NED-MS on C. albicans in conjunction with the PCS. DS dipropionate alone (10 µg/ml), the PCS alone, and DS dipropionate (10 µg/ml) combined with the PCS caused significant suppression of growth. Suppression was usually greatest in the early readings, with DS dipropionate causing about 30% inhibition, the PCS causing 40% inhibition, and the combination of the two causing 58% inhibition after 14.5 hr of growth. After 14.5 and 16 hr the combination caused more suppression of growth than did either component alone. Between 18 and 22 hr the combination caused more suppression than the PCS alone, but less than the DS dipropionate alone.

NED-MS, the PCS, and the combination of the two also caused significant suppression of the growth of C. albicans (Table 19). After 16 hr of growth NED-MS alone caused 27% inhibition, the PCS 41%, and the combination 49%. In all cases the combination of NED-MS and the PCS caused more suppression than did either alone.

TABLE 17

Effect of the plasma combination of steroids (PCS) on various microbes [a]

| | % inhibition at various hours [b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Organism | 6 | 8 | 10 | 12 | 13 | 14 | 16 | 18 |
| *C. albicans* | - | - | - | - | - | 48 | 30 | 17 |
| *S. pyogenes* | 100 | 90 | 54 | - | - | - | - | - |
| *S. aureus* | | | | | | | | |
| Serotype IX | - | - | - | 34 | 26 | 21 | - | - |
| Serotype X | - | - | - | 62 | 48 | 32 | - | - |
| Serotype XII | - | - | - | 10 | 10 | 8 | - | - |

[a] Experimental conditions were the same as in Table 4 for *C. albicans*, Table 6 for *S. pyogenes*, and Table 22 for *S. aureus*. Mean values obtained in 3 trials are given.

[b]

$$\% \text{ inhibition} = \frac{\text{turbidity of control - turbidity of PCS}}{\text{turbidity of control}}$$

TABLE 18

Effect of DS dipropionate on C. albicans, in the presence and absence of the PCS [a]

| Trial | Additions (μg/ml) | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 14.5 | 16 | 18 | 20 | 22 |
| 1 | Ethanol | 0 | 50 | 115 | 235 | 300 | 320 |
| | DS di, 10 | 0 | 40 | 70 | 130 | 170 | 230 |
| | PCS | 0 | 25 | 75 | 205 | 260 | 295 |
| | PCS&DSdi | 0 | 25 | 60 | 140 | 200 | 255 |
| 2 | Ethanol | 0 | 60 | 125 | 265 | 305 | 320 |
| | DSdi, 10 | 0 | 35 | 60 | 110 | 150 | 200 |
| | PCS | 0 | 30 | 80 | 210 | 265 | 305 |
| | PCS&DSdi | 0 | 15 | 60 | 150 | 185 | 245 |
| 3 | Ethanol | 0 | 45 | 90 | 215 | 280 | 310 |
| | DSdi, 10 | 0 | 30 | 55 | 115 | 155 | 205 |
| | PCS | 0 | 25 | 75 | 175 | 245 | 290 |
| | PCS&DSdi | 0 | 25 | 55 | 135 | 185 | 245 |
| Mean | Ethanol | 0 | 52 | 110 | 238 | 295 | 317 |
| | DSdi, 10 | 0 | 35 | 62 | 118 | 158 | 212 |
| | PCS | 0 | 27 | 77 | 197 | 257 | 297 |
| | PCS&DSdi | 0 | 22 | 58 | 142 | 190 | 248 |

[a] Experimental conditions were the same as in Table 4.

TABLE 19

Effect of NED and MS on C. albicans in the presence and absence of the PCS [a]

| Trial | Additions (µg/ml) | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 14.5 | 16 | 18 | 20 | 22 |
| 1 | Ethanol | 0 | 35 | 85 | 215 | 315 | 355 |
| | NED,10,MS,1 | 0 | 40 | 65 | 185 | 290 | 340 |
| | PCS | 0 | 30 | 70 | 190 | 280 | 330 |
| | PCS,NED,MS | 0 | 15 | 55 | 160 | 260 | 315 |
| 2 | Ethanol | 0 | 40 | 80 | 205 | 320 | 365 |
| | NED,10,MS,1 | 0 | 20 | 35 | 110 | 230 | 315 |
| | PCS | 0 | 15 | 45 | 135 | 245 | 315 |
| | PCS,NED,MS | 0 | 10 | 30 | 100 | 215 | 295 |
| 3 | Ethanol | 0 | 20 | 55 | 130 | 265 | 350 |
| | NED,10,MS,1 | 0 | 5 | 30 | 80 | 215 | 295 |
| | PCS | 0 | 15 | 45 | 95 | 240 | 330 |
| | PCS,NED,MS | 0 | 0 | 25 | 65 | 190 | 280 |
| Mean | Ethanol | 0 | 32 | 73 | 183 | 300 | 357 |
| | NED,10,MS,1 | 0 | 20 | 53 | 140 | 255 | 325 |
| | PCS | 0 | 18 | 43 | 125 | 245 | 317 |
| | PCS,NED,MS | 0 | 8 | 37 | 108 | 222 | 297 |

[a] Experimental conditions were the same as in Table 4.

Table 20 shows the effects of DS and the PCS on S. pyogenes, and Table 21 shows the effects of NED-MS and the PCS on the same organism. All steroids studied had strong suppressive effects on the growth of S. pyogenes, except NED-MS alone. It has already been seen that the PCS affected S. pyogenes more than any other microbe studied (Table 17), and it is evident from the data in Table 20 that 2.5 μg of DS per ml severely inhibited growth, particularly early in the experiment. The combination of the PCS and DS acted synergistically causing almost complete inhibition of growth. NED-MS caused barely significant inhibition of growth, and the combination of the PCS and NED-MS actually caused less suppression of growth than did the PCS alone.

The same steroids caused a wide range of effects on the three S. aureus serotypes studied (Tables 22-24), but all caused significant suppression of growth for all the serotypes. In all cases but one, the combination of the PCS with either DS or NED-MS caused more inhibition of growth than did the PCS, DS, or NED-MS alone. When Serotype X was studied, DS (1 μg/ml) produced virtually the same inhibition alone or in combination with the PCS, even though the PCS was very active (Table 23).

TABLE 20

Effect of DS on S. pyogenes in the presence and absence of the PCS[a]

| Trial | Additions (µg/ml) | Klett readings at various hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 6 | 7 | 8 | 10 | 22 |
| 1 | Ethanol | 0 | 40 | 80 | 100 | 100 | 100 |
| | DS,2.5 | 0 | 0 | 5 | 10 | 20 | 85 |
| | PCS | 0 | 0 | 5 | 10 | 45 | 100 |
| | PCS&DS | 0 | 0 | 0 | 0 | 0 | 10 |
| 2 | Ethanol | 0 | 20 | 60 | 95 | 95 | 95 |
| | DS,2.5 | 0 | 0 | 0 | 5 | 15 | 75 |
| | PCS | 0 | 0 | 10 | 20 | 90 | 100 |
| | PCS&DS | 0 | 0 | 0 | 0 | 0 | 10 |
| 3 | Ethanol | 0 | 20 | 50 | 95 | 100 | 110 |
| | DS,2.5 | 0 | 0 | 0 | 0 | 0 | 80 |
| | PCS | 0 | 0 | 0 | 0 | 0 | 95 |
| | PCS&DS | 0 | 0 | 0 | 0 | 0 | 0 |
| Mean | Ethanol | 0 | 27 | 63 | 97 | 98 | 102 |
| | DS,2.5 | 0 | 0 | 2 | 5 | 12 | 80 |
| | PCS | 0 | 0 | 5 | 10 | 45 | 98 |
| | PCS&DS | 0 | 0 | 0 | 0 | 0 | 7 |

[a] Experimental conditions were the same as in Table 6.

TABLE 21

Effect of NED and MS on S. pyogenes in the presence and absence of the PCS [a]

| Trial | Additions (µg/ml) | Klett readings at various hours | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 6 | 7 | 8 | 9 |
| 1 | Ethanol | 0 | 25 | 60 | 90 | 100 |
| | NED,10,MS,1 | 0 | 20 | 40 | 85 | 85 |
| | PCS | 0 | 0 | 0 | 0 | 0 |
| | PCS,NED,MS | 0 | 0 | 0 | 0 | 0 |
| 2 | Ethanol | 0 | 20 | 45 | 85 | 100 |
| | NED,10,MS,1 | 0 | 10 | 20 | 70 | 90 |
| | PCS | 0 | 0 | 0 | 0 | 0 |
| | PCS,NED,MS | 0 | 0 | 0 | 10 | 15 |
| 3 | Ethanol | 0 | 20 | 50 | 95 | 100 |
| | NED,10,MS,1 | 0 | 15 | 30 | 70 | 95 |
| | PCS | 0 | 0 | 0 | 0 | 0 |
| | PCS,NED,MS | 0 | 0 | 0 | 10 | 15 |
| Mean | Ethanol | 0 | 22 | 52 | 90 | 100 |
| | NED,10,MS,1 | 0 | 15 | 30 | 75 | 90 |
| | PCS | 0 | 0 | 0 | 0 | 0 |
| | PCS,NED,MS | 0 | 0 | 0 | 7 | 10 |

[a] Experimental conditions were the same as in Table 6.

TABLE 22

Effect of DS and NED-MS on S. aureus

(Serotype IX) in the presence and absence of the PCS

| Additions (μg/ml) | Klett readings at various hours | | | | |
|---|---|---|---|---|---|
| | 0 | 11 | 12 | 13 | 14 |
| Ethanol | 0 | 110 | 195 | 278 | 338 |
| DS, 1.0 | 0 | 48 | 97 | 150 | 198 |
| NED, 10, MS, 1 | 0 | 28 | 72 | 150 | 227 |
| PCS | 0 | 68 | 127 | 203 | 267 |
| PCS & DS | 0 | 13 | 32 | 70 | 106 |
| PCS & NED-MS | 0 | 15 | 33 | 77 | 130 |

[a] Experimental conditions were the same as in Table 6, except that Casein Hydrolysate Broth and the blue filter of the colorimeter were used. Values given are the mean of three separate trials.

TABLE 23

Effect of DS and NED-MS on S. aureus

(Serotype X) in the presence and the absence of the PCS [a]

| Additions (µg/ml) | Klett readings at various hours | | | | |
|---|---|---|---|---|---|
| | 0 | 11 | 12 | 13 | 14 |
| Ethanol | 0 | 127 | 197 | 233 | 262 |
| DS, 1.0 | 0 | 20 | 43 | 78 | 120 |
| NED,10,MS,1 | 0 | 102 | 165 | 215 | 257 |
| PCS | 0 | 33 | 73 | 121 | 177 |
| PCS & DS | 0 | 20 | 53 | 87 | 140 |
| PCS & NED-MS | 0 | 5 | 13 | 30 | 58 |

[a] Experimental conditions were the same as in Table 22.

TABLE 24

Effects of DS and NED-MS on S. aureus

(Serotype XII) in the presence and absence of the PCS [a]

| Additions (µg/ml) | Klett readings at various hours | | | | |
|---|---|---|---|---|---|
| | 0 | 11 | 12 | 13 | 14 |
| Ethanol | 0 | 140 | 213 | 298 | 308 |
| DS, 1.0 | 0 | 57 | 103 | 167 | 202 |
| NED,10,MS,1 | 0 | 116 | 183 | 258 | 272 |
| PCS | 0 | 113 | 190 | 268 | 282 |
| PCS & DS | 0 | 48 | 90 | 148 | 182 |
| PCS & NED-MS | 0 | 100 | 162 | 228 | 260 |

[a] Experimental conditions were the same as in Table 22.

# IV. DISCUSSION

## A. Effects of DS and MED-MS on C. albicans and S. pyogenes.

An examination of the literature reveals that much work has been done on the effects of DS, NED, and MS on S. aureus. The growth of S. aureus has been shown to be suppressed by all three steroids (Baman, 1968; Yotis, 1967). Other cellular functions, such as respiration and the production of macromolecules, have also been studied in the presence of DS (Yotis and Baman, 1969; Yotis and Yotis, 1970). When DS, NED, and MS were found to cause an increase in the leakage of cellular materials (Yotis and Bhattacharyya, 1971) and to bind to mucopeptides (Yotis and Fitzgerald, 1974), it was proposed that they influenced staphylococci by exerting an effect on the cell envelope. It is of interest, therefore, to compare our results with those of other researchers for S. aureus, to determine whether similar effects possibly occur in C. albicans and/or S. pyogenes.

Although one paper has been published on the effects of NED and NED-MS on the growth of S. aureus, most of the data obtained was from cells growing under anaerobic conditions (Yotis, 1967). Since our experiments were performed aerobically, no valid comparisons of the effect of NED and NED-MS on growth can be made.

The concentrations of DS required to suppress the growth of S. aureus are as low as 1 μg per ml of media (Baman, 1968) under conditions basically similar to those used in the present research. Baman also found that concentrations of 6.0 to 7.3 μg of DS per ml completely inhibited the growth of

S. aureus (Serotypes I through XII) for 29 hr. No data obtained under similar conditions could be found in the literature for DS dipropionate.

The data in Table 9 revealed that higher concentrations of DS were necessary to suppress the growth of C. albicans than to suppress the growth of S. aureus. Even higher amounts of DS dipropionate were required to suppress growth of C. albicans, (Table 8), but this difference could be attributed to a difference in steroid activity rather than the organism used.

Concentrations of DS ranging from 1 to 5 μg per ml had basically the same effect on S. pyogenes (Table 10) as they did on S. aureus.

Growth studies with S. aureus revealed a definite pattern in the effect of DS. DS had been shown to have an immediate effect on growth, causing an extended lag phase or, in amounts of 5 to 10 μg, an immediate decrease in viable units. Cells then recovered from this effect and entered the exponential phase with a growth rate approximately equal to that of control cells. They entered stationary phase later than did the control cells but reached almost similar cellular concentrations (Baman, 1968).

When the growth of C. albicans was studied (as in Table 8), it appeared initially that the same pattern might exist because the measurements were only made of late growth. Measuring growth by viable cell counts allowed the entire growth curve to be studied. These data revealed that an immediate effect did not occur (Fig. 1). Instead, growth was abruptly inhibited by DS dipropionate in mid-exponential phase. This seemed to be an important difference between the growth of C. albicans and S. aureus in the presence of a steroid, and it was therefore investigated further (Table 15 and Fig. 4-5). Cells were affected at different stages in growth, rather than only in lag

phase as were staphylococci, when varying amounts of DS were used. Furthermore, C. albicans did not immediately absorb labeled DS, while staphylococci abosrbed labeled DS within 15 minutes (Yotis and Baman, 1970).

The pattern in the suppression of the growth of S. pyogenes by DS was quite similar to that of S. aureus, as determined in both turbidimetric and viable unit studies (Table 10 and Fig. 3).

Baman (1968) found that DS concentrations as low as 5 µg per ml significantly reduced the exogeneous respiration of S. aureus.

DS dipropionate did not change the oxygen consumption of C. albicans (Table 11). Other investigators using yeast found that at certain concentrations, DS actually increased oxygen consumption (Schacter, 1953; Salmony, 1956). Thus the steroids apparently do not affect the respiration of fungi in the same manner as they do the respiration of staphylococci.

Results from the present studies of the influence of DS on the respiration of S. pyogenes (Table 12), compare favorably with those from staphylococcal studies. Oxygen consumption was low, but DS seemed to inhibit respiration.

Fitzgerald (1971) and Baman (1968) had shown that 5 to 20 µg of DS enhanced the leakage of labeled materials from S. aureus. Under precisely the same conditions, however, DS dipropionate did not enhance leakage in C. albicans (Tables 13-14). Similar studies were attempted with S. pyogenes, but, as discussed previously, no conclusions could be drawn from these studies.

In summary, it is evident that there are major differences between the effects of DS dipropionate and DS on C. albicans and the effects of DS on

S. aureus. Such differences do not appear in the effects of DS on S. pyogenes.

The growth, respiration, and permeability of C. albicans were not affected in the same manner by steroids as are these functions of S. aureus. These differences may be the result of an entirely different primary mode of action, i.e., other than membrane effects, or they may reflect an alternate method of absorption. Since our data (Table 15 and Fig. 4-6) show that C. albicans must grow in TSB to absorb DS, it is not surprising that DS dipropionate did not affect C. albicans in the manometric, permeability, and fractionation studies. In all three of these studies, DS dipropionate was added to resting cells in buffer. It is possible that if these studies could be performed on cells which were actively growing in DS an effect would be observed. Such studies might show that once C. albicans had absorbed DS it would be affected in the same manner as S. aureus.

It is difficult to speculate on why growth in TSB causes C. albicans to be susceptible to DS, due to the many changes that could occur in cells during that growth. Studies such as the comparative effects of many steroids in TSB, the effects of steroids in other media, the binding locations of the steroids, etc., may reveal the cause.

Investigation of two aspects of the data presented in this report could also lead to an explanation. First, why do different concentrations of DS affect the cells at different stages of growth? Second, since cells for all growth studies were first subcultured in TSB, wouldn't an immediate effect always be seen?

The differences found between the effects of DS on C. albicans and the effects of DS on S. aureus are supported by the differences in the cell structure and metabolism of the two organisms. First, DS had been found to bind to the mucopeptide of the S. aureus cell wall (Yotis and Fitzgerald, 1974). Fungal cell walls contain polysaccharides, but they are different in nature from mucopeptide. Thus, the binding of DS would necessarily be different in a fungus. Second, the vast differences between a prokaryotic cell (S. aureus) and a eukaryotic cell (C. albicans) might account for a difference in the mode of action of DS on S. aureus and C. albicans. Third, bacterial cells neither contain nor require steroids, but certain steroids are important components of the yeast cell membrane. It is possible that DS interferes with the placement of the proper steroids in the yeast cell membranes, rather than binding illegitimately as it does in the bacterial cell envelope.

The similarities found in the effects of DS on S. pyogenes and S. aureus are supported by the similarities in the cell structures of these two organisms. Since both S. aureus and S. pyogenes are gram-positive, coccal forms of bacteria, the similarities in the cell wall structure and cellular functions preclude a similarity in the mode of action of DS on the two organisms.

B. Effects of DS and NED-MS in Conjunction with the PCS.

The PCS alone caused significant suppression of the growth of each of the organisms studied. Although percent inhibition varied widely, it did not cause complete inhibition of growth for any microbe. The fact that these steroids, present in normal plasma, suppress the growth of pathogenic microorganisms poses some interesting questions. Why would pathogens be sensitive to a factor with which they are often associated? Do steroids play an important role in host-parasite relationships? These and other questions deserve further study.

When DS or NED-MS were added to the PCS, there were basically three possibilities for the combined effect. First, the total effect could have been the simple addition of individual effects. Second, enhancement might have occurred, with the total effect being larger than the simple addition of individual effects. Third, some of the individual effects could have been lost and the total effect less than that by simple addition.

Examination of our data (Tables 18-24) reveals that the third possibility occurred; however, the total effect was usually greater than that of the PCS or the additional steroid alone. In no case did a total loss of either effect occur.

The fact that a loss of effect did not occur is important when attempting to correlate *in vitro* studies of the suppressive effects of steroids with *in vivo* studies or clinical findings. If the PCS would have nullified the effects of DS or NED-MS, then the addition of these pharmaceutical steroids to a patient's serum would have no effect on host-parasite relationships. The data here, however, clearly suggests that DS or NED-MS may have a direct effect on pathogenic microbes in an *in vivo* situation.

The clinical finding that oral contraceptives may cause an increase in yeast vaginitis (Walsh et al., 1968) is an example of the effects that steroids may have on host-parasite relationships. Our studies indicate that sex steroids may act differently on C. albicans than they do on gram positive bacteria. If so, this difference might give C. albicans an ecological advantage over other microbes when high concentrations of sex steroids are present. The result would be an overgrowth of yeast such as that found when some patients are receiving antibiotics directed against bacteria. This and other clinical correlations are, of course, highly speculative, but it would seem that they are worthy of attention.

# V. SUMMARY

Norethindrone (NED) and mestranol (MS) were found to suppress the growth of Candida albicans in concentrations of 10 µg of NED and 1 µg of MS per ml of media. NED and MS had little effect on the growth of Streptococcus pyogenes. Diethylstilbestrol (DS) dipropionate (5-20 µg/ml) and DS (7.5-10 µg/ml) suppressed the growth of C. albicans, and DS (1-5 µg/ml) suppressed the growth of S. pyogenes. Whereas the growth of S. pyogenes was immediately affected by DS, the growth of C. albicans was not. It was found that prior growth in TSB for short intervals (4 hr) caused the growth of C. albicans to be immediately inhibited by DS. DS dipropionate did not affect either the respiration or the release of cellular constituents from C. albicans, but DS suppressed the respiration of S. pyogenes. NED-MS, DS, and DS dipropionate usually suppressed the growth of C. albicans, S. pyogenes, and Staphylococcus aureus both in the presence and the absence of a combination of steroids found in normal human plasma.

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APPROVAL SHEET

The thesis submitted by John N. Haan has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

November 14, 1974
Date

William W. Yates
Signature of Advisory Committee Director

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